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Dibenzocyclooctadiene lignans, gomisins J and N inhibit the Wnt/β-catenin signaling pathway in HCT116 cells

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ABSTRACT

Here, we report that gomisin J and gomisin N, dibenzocyclooctadiene type lignans isolated from *Schisandra chinensis*, inhibit Wnt/β-catenin signaling in HCT116 cells. Gomisins J and N appear to inhibit Wnt/β-catenin signaling by disrupting the interaction between β-catenin and its specific target DNA sequences (TCF binding elements, TBE) rather than by altering the expression of the β-catenin protein. Gomisins J and N inhibit HCT116 cell proliferation by arresting the cell cycle at the G0/G1 phase. The G0/G1 phase arrest induced by gomisins J and N appears to be caused by a decrease in the expression of Cyclin D1, a representative target gene of the Wnt/β-catenin signaling pathway, as well as Cdk2, Cdk4, and E2F-1. Therefore, gomisins J and N, the novel Wnt/β-catenin inhibitors discovered in this study, may serve as potential agents for the prevention and treatment of human colorectal cancers.

1. Introduction

The Wnt/β-catenin signaling pathway is a crucial pathway regulating cell proliferation, differentiation, migration, survival, and death. This pathway has been implicated in the initiation and progression of various human cancers including colorectal cancer [1,2]. Therefore, inhibitors of the Wnt/β-catenin signaling pathway may be valuable candidates for use as cancer chemopreventive or chemotherapeutic agents. Naturally occurring dietary compounds are the most attractive candidates. Flavonoids are the best known natural products that inhibit Wnt signaling; for example, EGCG, quercetin, fisetin, and genistein are reported to be Wnt inhibitors [1–3]. In addition to flavonoids, curcumin (a major polyphenolic compound of turmeric), resveratrol (a polyphenol from grape), lupeol (a dietary triterpene), and retinoids are known to inhibit the Wnt/β-catenin signaling pathway and to function as cancer chemopreventive agents [1,2]. Lignans are another major class of natural products that are well-known for their cancer chemopreventive potential [4,5]. However, the mechanisms by which lignans inhibit Wnt/β-catenin signaling remain poorly understood [6,7].

Schisandra chinensis is a woody vine that grows in Korea, China, Japan, and Russia. The dried fruit of *S. chinensis* has been consumed as tea, wine, and a traditional phytomedicine [8]. Recently, the dibenzocyclooctadiene lignans isolated from the fruit of *S. chinensis* have been suggested for use as potential cancer chemopreventive agents because they possess anti-inflammatory, antioxidant, antiproliferative, and proapoptotic activities [9–12]. To date, however, the mechanisms by which the dibenzocyclooctadiene lignans from *S. chinensis* inhibit Wnt/β-catenin signaling have not been investigated.

The purpose of the present study was to answer a simple question: whether dibenzocyclooctadiene lignans from *S. chinensis* inhibit Wnt/β-catenin signaling in human colorectal cancer. The effects of these lignans were evaluated by treating cells with each lignan and then using a luciferase reporter assay to measure β-catenin/TCF transcriptional activity. The ability of β-catenin to bind its TCF binding element after lignan treatment was also determined. We also investigated the cell cycle distribution, apoptosis, and changes in molecular signaling in HCT116 cells treated with these dibenzocyclooctadiene lignans.

2. Materials and methods

2.1. Materials

Dibenzocyclooctadiene lignans, including gomisin J and gomisin N, were isolated from the fruit of *S. chinensis* as previously described [13]. Propidium iodide (PI) and dimethyl sulfoxide (DMSO) were purchased from Sigma (St. Louis, MO). Gomisins J and N were dissolved in DMSO for *in vitro* experiments. Antibodies against Cyclin D1, Cdk4, E2F-1, PARP, cleaved caspase-3, and β-catenin were purchased from Cell Signaling Technology (Danvers, MA).

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Antibodies against Cdk2 and β-actin were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Secondary anti-rabbit and anti-mouse antibodies were purchased from Santa Cruz Biotechnology.

2.2. Cell culture

HCT116 cells were obtained from the American Type Culture Collection (Rockville, MD). HCT116 cells were cultured in Minimal Essential Medium supplemented with 10% fetal bovine serum, 100 units/ml penicillin, and 100 μg/ml streptomycin. Cells were cultured in a humidified atmosphere (95% air, 5% CO_2) at 37 °C.

2.3. Luciferase assay

To measure β-catenin/TCF transcriptional activity, we performed a luciferase reporter assay (reporter plasmids; TOPFlash, FOPFlash, and pRL-CMV) as described previously [6].

2.4. Measurement of antiproliferative activity

Cell viability was determined by measuring mitochondrial dehydrogenase activity as previously described [4,14]. For the clonogenic assay, HCT116 cells (1000 cells per well) were seeded in a 24-well plate and incubated for 24 h. Cells were then treated with gomisin J or N every 3–4 days. Seven days after treatment, the cell colonies were stained with Coomassie Brilliant Blue R-250 as previously described [15].

2.5. Flow cytometric DNA content analysis

Cell cycle profiles were obtained by flow cytometric DNA content analysis as previously described [4,14].

2.6. Measurement of phosphatidylserine externalization

Apoptotic cell death was evaluated by measuring the externalization of phosphatidylserine using flow cytometric analysis of cells stained with annexin V-fluorescein and PI, as previously described [14].

2.7. Observation of morphological changes

The cellular morphology was observed by phase contrast microscopy using an OLYMPUS CK40 microscope (Tokyo, Japan) as previously described [14].

2.8. Oligonucleotide pull-down assay

HCT116 cells (5×10^5) were seeded in 60-mm dishes, incubated for 24 h, and then treated with gomisin J or N for 48 h. The cells were then lysed in Lysis buffer as previously described [4,15]. Total cell lysates were incubated with 1 mg of biotinylated double-stranded oligonucleotides in HKMG buffer (10 mM HEPES, pH 7.9, 100 mM KCl, 5 mM $MgCl_2$, 10% glycerol, 1 mM DTT, and 0.5% NP-40) for 16 h. The oligonucleotide sequences are listed as follows (only the nucleotide sequences of the sense strands are shown): 5′-AAGATCAAAGGGGGTAAGATCAAAGGGGGTAAGATCAAAGGG-3′. To anneal the oligomers, the sense and antisense oligomers were mixed and incubated in reaction buffer (50 mM Tris–HCl, pH 8.0, 1 mM $MgCl_2$, and 100 mM NaCl) at 95 °C for 5 min. To collect DNA-bound proteins, the mixtures were incubated with streptavidin-agarose beads (Thermo, Rockford, IL) for 6 h, washed three times with HKMG buffer and precipitated by centrifugation at 1000*g* for 5 min. The precipitate was analyzed by Western blot as previously described [4,15].

2.9. Statistical analysis

The data are expressed as the mean ± standard deviation (SD) or standard error of the mean (SEM). Statistical analyses were performed by one-way analysis of variance (ANOVA) followed by Dunnett's multiple comparison test using GraphPad Prism 5 software (La Jolla, CA).

3. Results

3.1. Gomisins J and N inhibit the transcriptional activity of β-catenin/TCF

To evaluate the inhibitory activity of nine dibenzocyclooctadiene lignans isolated from *S. chinensis* (gomisin J, gomisin N, schisandrin A, schisandrin B, schisandrin C, schisandrol A, schisandrol B, tigloylgomisin H, and angeloylgomisin H) on Wnt/β-catenin signaling in HCT116 cells [13], we transiently co-transfected cells with reporter plasmids (TOPFlash, FOPFlash, and pRL-CMV) and used a luciferase reporter assay to measure β-catenin/TCF transcriptional activity. Of the nine dibenzocyclooctadiene lignans evaluated, only gomisin J and gomisin N (chemical structures shown in Fig. 1A) significantly inhibited β-catenin/TCF transcriptional activity in a dose-dependent manner (Fig. 1B).

3.2. Gomisins J and N disrupt the binding of β-catenin to TCF binding elements

HCT116 cells contain a mutated β-catenin that does not undergo phosphorylation by GSK3β and therefore is not degraded by the proteasome system [16]. Accordingly, neither gomisin J or N decrease the expression of total β-catenin in these cells, and we were able to use an oligonucleotide pull-down assay to measure the binding of β-catenin to specific DNA sequences, called TCF binding elements (TBE), after treatment with gomisin J or N. Gomisin J slightly decreased the binding of β-catenin to TBE. Moreover, gomisin N significantly inhibited the binding of β-catenin to TBE (Fig. 1C and D). These results suggest that both gomisins J and N inhibit the Wnt/β-catenin signaling pathway by inhibiting β-catenin binding to TBE.

3.3. Gomisins J and N inhibit the proliferation of HCT116 cells

We next evaluated whether gomisins J and N inhibit the proliferation of HCT116 cells. A cell viability assay that measures cellular mitochondrial dehydrogenase activity suggested that both gomisin J and gomisin N possess potent antiproliferative activity in HCT116 cells. The antiproliferative activity of gomisin N was greater than that of gomisin J (Fig. 2A). Similar results were obtained in the SW480 human colon cancer cells (data not shown). We confirmed the antiproliferative activities of gomisins J and N by performing a clonogenic assay in HCT116 cells. Seven days of continuous treatment with gomisin J or N significantly decreased colony formation in dose-dependent manner (Fig. 2B and C). These results suggest that gomisins J and N, which inhibit the Wnt/β-catenin signaling pathway, also actually inhibit the proliferation of HCT116 human colon cancer cells.

3.4. Gomisins J and N induce cell cycle arrest at the G0/G1 phase

We next evaluated the cell cycle distribution of gomisin-treated cells using flow cytometric DNA content analysis. Both gomisin J and gomisin N induce a dose-dependent accumulation of cells in the G0/G1 phase, which is accompanied by a decrease in the proportion of cells in S phase. Treatment with 100 μM gomisin J or

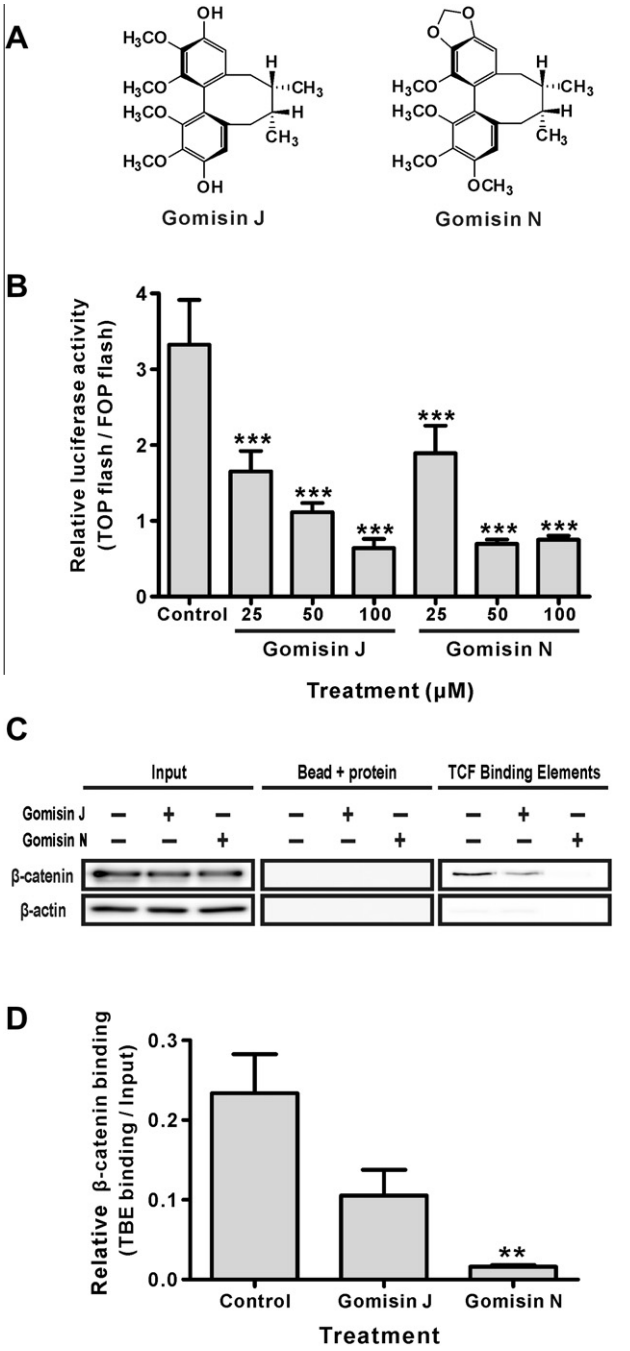

Fig. 1. Gomisins J and N inhibit the TCF/β-catenin-dependent transcriptional activity and β-catenin binding to the TCF binding element (TBE). (A) The chemical structures of gomisins J and N. (B) HCT116 cells were transfected with luciferase reporter plasmids and then treated with indicated concentrations of gomisin J or N for 48 h. The relative luciferase activity was calculated by dividing TOPFlash activity by FOPFlash activity. Values shown are mean ± SD from three replicates. $^{***}p < 0.001$ compared to control. The graphs shown are representative of three independent experiments. (C) The binding of β-catenin to TBE was measured using the oligonucleotide pull-down assay. HCT116 cells were treated with gomisin J or N (100 μM) for 48 h. (D) Relative β-catenin binding was calculated by normalizing the TBE bound β-catenin to the total β-catenin input. Values shown are mean ± SEM of three independent experiments. $^{**}p < 0.01$ compared to control.

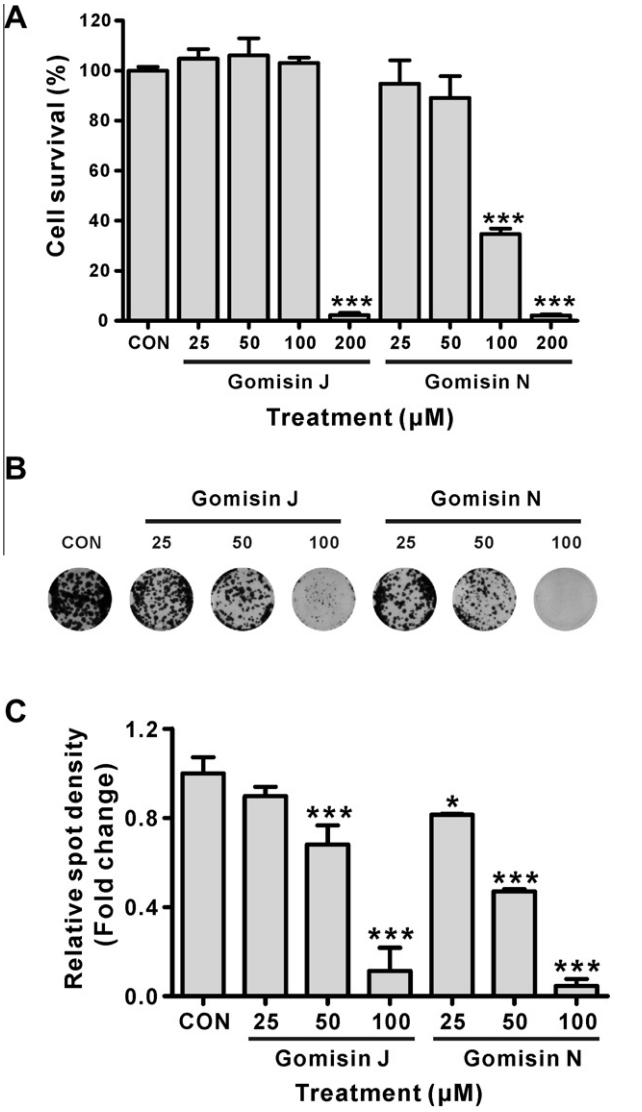

Fig. 2. Gomisins J and N inhibit HCT116 cell proliferation. (A) Cell survival was determined using a cell viability assay. HCT116 cells were treated with indicated concentrations of gomisin J or N for 48 h. Values shown are mean ± SD from three replicates. $^{***}p < 0.001$ compared to control (CON). The data shown are representative of three independent experiments. (B) Clonogenic assay. HCT116 cells were treated with gomisin J or N for 7 days. The images shown are representative of three independent experiments. (C) Relative spot density was calculated as the fold-increase relative to control. Values shown are mean ± SD from three replicates. $^{*}p < 0.05$ and $^{***}p < 0.001$ compared to control.

N significantly increased the percentage of cells in the G0/G1 phase, to 75.34 ± 4.35 and 70.65 ± 15.40, respectively, whereas the percentage of vehicle control cells in the G0/G1 phase remained at 48.43 ± 10.85 (Fig. 3A). Based on this result, we speculated that the antiproliferative effects of gomisins J and N result from the induction of cell cycle arrest at the G0/G1 phase.

3.5. Gomisin N induces apoptosis, but gomisin J does not induce apoptosis

We also investigated the proapoptotic activity of gomisins J and N in HCT116 cells. Using flow cytometric DNA content analysis, we measured the percentage of cells with sub G0/G1 content (%), which is an indirect indicator of apoptosis. Compared with the vehicle control, gomisin N (100 μM) significantly increased the proportion of cells with sub G0/G1 content; in contrast, gomisin J did not alter the sub G0/G1 population (Fig. 3B). To confirm this result, we measured the externalization of phosphatidylserine, a precise indicator of apoptosis, in HCT116 cells after treatment with gomisin J or N. The early apoptotic cell population (annexin V-fluorescein-positive and PI fluorescence-negative) increased to 5.20%

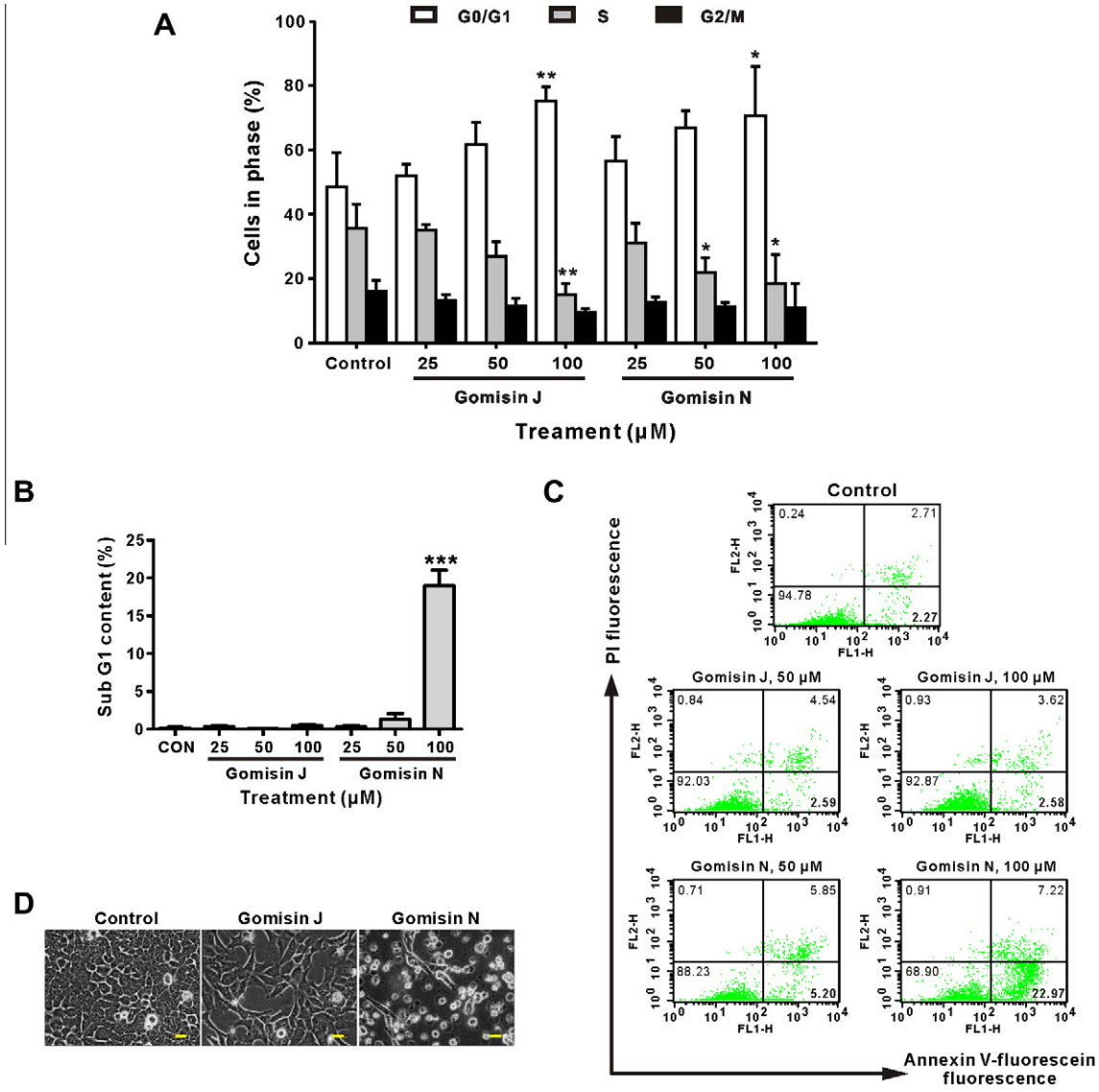

Fig. 3. The effects of gomisins J and N on the cell cycle distribution and apoptosis induction. HCT116 cells were treated with indicated concentrations of gomisin J or N for 48 h. (A) Cell cycle distribution and (B) the sub G1 population (%) were evaluated by flow cytometric DNA content analysis. Values shown are mean ± SD of three independent experiments. $^{*}p < 0.05$, $^{**}p < 0.01$, and $^{***}p < 0.001$ for the percentage of cells in each cell cycle phase compared with control (CON). (C) The externalization of phosphatidylserine was evaluated by flow cytometric analysis after annexin V-fluorescence and PI staining. The percentage of the cell population in each quadrant is shown. The lower right quadrant represents an early apoptotic cell population, and the percentage of cells in this quadrant is indicated in bold numbers. (D) Changes in cell morphology. The images are representative of at least three independent experiments (bar = 20 μm).

and 22.97% upon treatment with gomisin N at 50 or 100 μM, respectively. Cells treated with gomisin J at 50 or 100 μM, as well as those that received the vehicle control, possessed only 2.59%, 2.58%, and 2.27% early apoptotic cell populations, respectively (Fig. 3C). The microscopic observation of the morphological changes indicated that only gomisin N induced the characteristic morphological changes associated with apoptosis, such as membrane blebbing, formation of apoptotic bodies (Fig. 3D), and chromatin condensation (data not shown). Taken together, these data indicate that only gomisin N, not gomisin J, induces apoptosis in HCT116 cells. Similar results were obtained in SW480 cells; only gomisin N induced apoptosis (data not shown). The results of the apoptosis assays are consistent with the antiproliferative activity of gomisins J and N. The strong antiproliferative activity of gomisin N likely results from its ability to induce both G0/G1 phase arrest and apoptosis, whereas gomisin J induced G0/G1 phase arrest but did not induce apoptosis.

3.6. Gomisins J and N inhibit the protein expression of Cyclin D1, a representative Wnt/β-catenin target gene

To elucidate the molecular mechanism underlying the G0/G1 phase arrest induced by gomisins J and N, we investigated the expression of cell cycle regulatory proteins using Western blot analysis. First, we measured the expression of Cyclin D1, which is a representative Wnt/β-catenin signaling target gene that regulates the G1 phase of the cell cycle [1,17]. Treatment with gomisin J (100 μM) slightly decreased the expression of Cyclin D1 in a dose-dependent manner, whereas gomisin N treatment (100 μM) significantly decreased the expression of Cyclin D1 protein (Fig. 4A and D). We also measured the protein expression of Cdk4 and Cdk2, which are important cyclin-dependent kinases that regulate G1 progression and G1/S transition, respectively, after treatment with gomisin J or N [17,18]. Both gomisins J and N decreased the protein expression of Cdk2 and Cdk4 significantly in

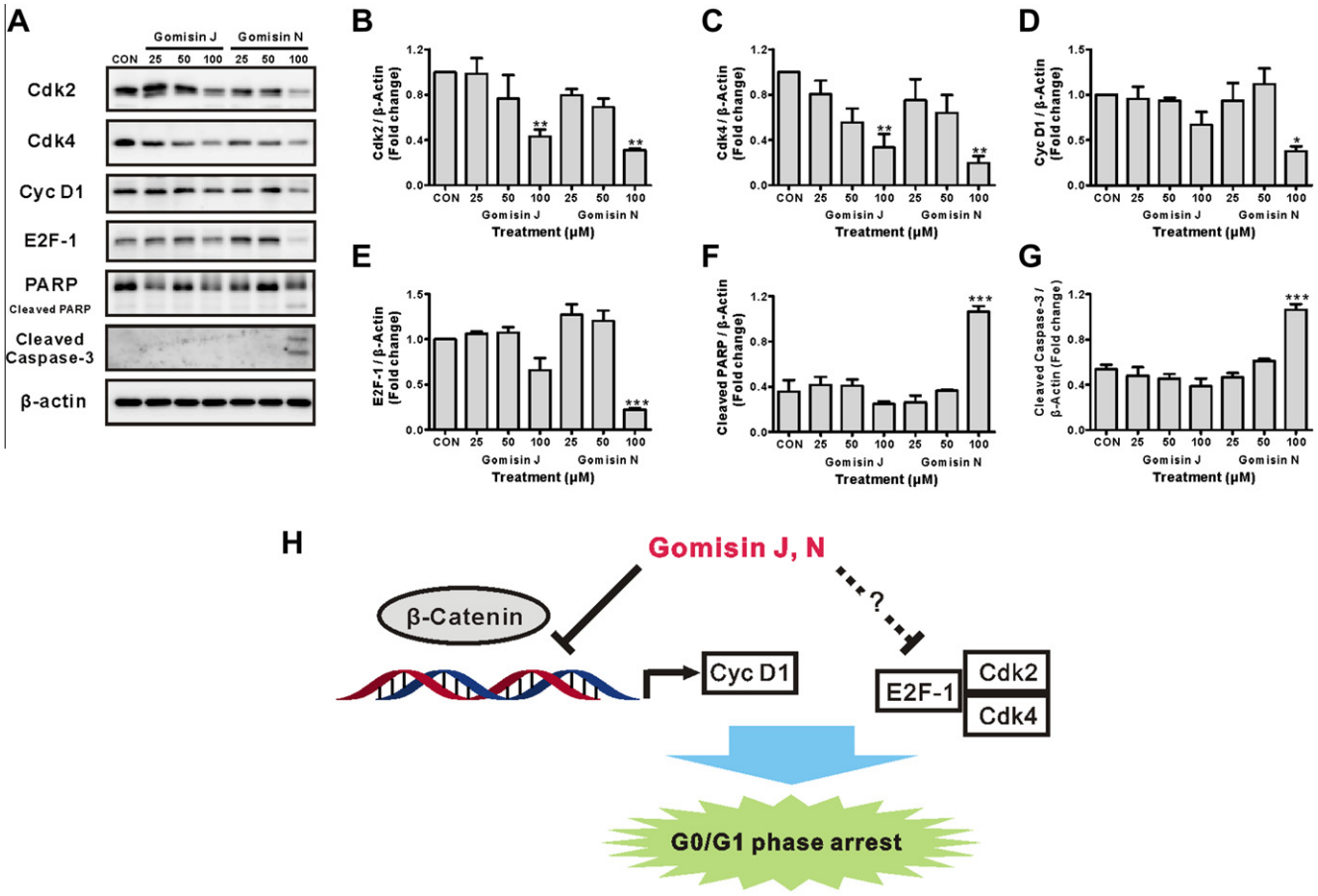

Fig. 4. The effects of gomisins J and N on the expression of cell cycle and apoptosis regulatory proteins. HCT116 cells were treated with indicated concentrations of gomisin J or N for 48 h. (A) The immunoblots shown are representative of three independent experiments. The relative expression of Cdk2/β-actin (B), Cdk4/β-actin (C), CycD1/β-actin (D), E2F-1/β-actin (E), cleaved PARP/β-actin (F), and cleaved caspase-3/β-actin (G) were determined by densitometry. Values shown are mean ± SEM from three independent experiments. $^{*}p < 0.05$, $^{**}p < 0.01$, and $^{***}p < 0.001$ compared to control (CON). (H) A schematic diagram of the possible molecular mechanism underlying the G0/G1-phase arrest induced by gomisins J and N in HCT116 cells.

a dose-dependent manner (Fig. 4A–C). The expression of E2F-1, an important transcription factor that regulates the G1/S cell cycle checkpoint [19], is also decreased by treatment with gomisins J and N (100 μM) (Fig. 4A and E). We believe that the decreased expression of Cdk2, Cdk4, and E2F-1, in addition to the decrease in Cyclin D1 expression, is the main cause of the G0/G1 phase arrest induced by gomisins J and N.

We also measured apoptotic protein expression in cells after treatment with gomisin J or N. Only 100 μM gomisin N significantly induced the appearance of cleaved caspase-3 and PARP (Fig. 4A, F and G), which are definitive markers of apoptosis induction. These data are consistent with the results of the apoptosis assays described in the previous section (Fig. 3B–D).

4. Discussion

Here, we present the first evidence that gomisins J and N, dibenzocyclooctadiene lignans isolated from the fruit of *S. chinensis*, inhibit the Wnt/β-catenin signaling pathway in human colon cancer cells. The Wnt/β-catenin signaling pathway is a critical pathway that regulates the initiation and progression of cancer; accordingly, inhibitors of the Wnt/β-catenin pathway have long been sought for use as cancer chemopreventive or chemotherapeutic agents. Natural products are particularly promising sources of Wnt inhibitors. Flavonoids (quercetin, fisetin, EGCG, and genistein), alkaloids (antofine, isoreserpine, and murrayafolin A), terpenoids (lupeol, koetjapic acid, and ganodermanontriol), and other dietary polyphenols (resveratrol, curcumin, and ellagic acid) have previously been reported to inhibit the Wnt/β-catenin pathway [1–3,20–23]. However, to date, the effects of lignans on Wnt/β-catenin signaling have been poorly understood. Two recent reports have described the regulation of the Wnt/β-catenin signaling pathway by plant lignans. In the first report, arctigenin, a dibenzylbutyrolactone lignan isolated from *Saussurea salicifolia*, inhibited SW480 cell proliferation via regulation of Wnt/β-catenin signaling [6,14]. In the second report, diphyllin, an arylnaphthalene lignan isolated from *Cleistanthus collinus*, was reported to inhibit V-ATPase and decreased the expression of β-catenin and its target genes c-myc and Cyclin D1 in SGC7901 human gastric cancer cells [7]. In the present study, we demonstrate that Wnt/β-catenin signaling is inhibited by gomisins J and N, which are dibenzocyclooctadiene type lignans isolated from *S. chinensis*.

The results of the present study can also be applied to the development of cancer chemopreventive agents. This is the first report that plant lignans isolated from the edible plant species *S. chinensis* inhibit Wnt/β-catenin signaling. We expect that gomisins J and N, as well as *S. chinensis* fruit extracts, might be useful dietary supplements for the prevention and treatment of human cancer because gomisins J and N are known to possess anti-inflammatory, antioxidant, antiproliferative, proapoptotic, and phase II detoxification enzyme induction activities [9–13], in addition to their Wnt/β-

catenin inhibitory activities. We also suggest that these dibenzocyclooctadiene lignans may serve as promising lead compounds for the development of Wnt inhibitors for cancer treatment as well as marker compounds for the standardization of *S. chinensis* extracts for use as a cancer chemopreventive agent.

Gomisins J and N inhibited the transcriptional activity of Wnt/β-catenin in HCT116 cells. These inhibitory activities resulted from the disruption of the binding between β-catenin and TCF binding elements (TBE) rather than a decrease in β-catenin expression (Fig. 1). We thought that the expression levels of the β-catenin protein did not change because HCT116 cells cannot degrade β-catenin due to their dysfunctional degradation system of β-catenin. HCT116 cells encode a mutant β-catenin that cannot be phosphorylated by GSK3β, thereby preventing its ubiquitination and subsequent proteosomal degradation [16]. Similarly, quercetin inhibited β-catenin/TCF transcriptional activity without decreasing the levels of β-catenin in SW480 cells [3], which have a truncated APC and dysfunctional β-catenin degradation system [16]. Ionomycin inhibits β-catenin/TCF transcriptional activity, but not by modulating the β-catenin degradation machinery, which is comprised of the Axin-APC-GSK complex in HCT116 cells [24]. Rather, the Wnt inhibitory activities of quercetin and ionomycin are mediated through a decrease in nuclear β-catenin and the disruption of TCF complexes on DNA binding elements [3,24]. TCF complexes are composed of transcription factors and coactivators such as β-catenin, various TCFs, LEF, CBP/p300, and other factors [25]. We speculate that the disruption of the interaction between β-catenin and TBE by gomisins J and N is caused by a loss or decrease in the expression of one or more components of the TCF complex or, alternatively, the binding of a transcriptional repressors such as Groucho, histone deacetylase [25], or another unknown repressor. To elucidate this mechanism, we will further analyze the TBE binding proteins after treatment with Wnt inhibitors, including gomisins J and N, using the oligonucleotide pull-down assay and liquid chromatography–mass spectroscopy (LC–MS/MS). However, our present results indicate that gomisins J and N could be exploited to treat or prevent human colon cancer regardless of the presence of mutations that disrupt the Axin-APC-GSK pathway controlling β-catenin degradation, which is commonly mutated in human colon carcinogenesis [16].

Gomisins J and N also inhibited the protein expression of Cyclin D1 (Fig. 4), a representative target gene of the Wnt/β-catenin signaling pathway. Cyclin D1 is a crucial G1 phase cell cycle regulatory protein [17]. We speculated that a decrease in the expression of Cyclin D1 resulted in the cell cycle arrest at the G0/G1 phase in HCT116 cells. Gomisins J and N also inhibited the protein expression of Cdk2 and Cdk4, which are important cyclin-dependent kinases that regulate the G1/S transition and G1 progression, respectively [17,18]. Additionally, gomisins J and N decreased the protein expression of E2F-1, an important transcription factor regulating the G1/S checkpoint [19]. Taken together, these data suggest that gomisins J and N induce G0/G1 cell cycle arrest via the inhibition of both G1 progression and the G1/S transition. A schematic diagram depicting the cell cycle arrest induced by gomisins J and N in HCT116 cells is shown in Fig. 4H.

Gomisin N, but not gomisin J, induced apoptosis in HCT116 cells (Fig. 3B–D). Previous studies indicate that gomisin N induces apoptosis in other human cancer cells, such as HepG2 human liver cancer cells and U937 human leukemia cells [10,26], as well as in colon cancer. The structure-proapoptotic activity relationships of dibenzocyclooctadiene lignans from *S. chinensis* and the detailed molecular mechanisms underlying dibenzocyclooctadiene lignan-induced apoptosis remain unclear, and further in-depth studies are needed [10,11,26]. Preliminary data generated using the H_2-DCFDA assay indicated that neither gomisin J nor gomisin N (25–100 μM) induced the accumulation of intracellular reactive oxygen species (ROS) in HCT116 cells (data not shown), indicating that apoptosis was not caused by the accumulation of ROS. Similarly, a previous study reported that deoxyschizandrin and γ-schizandrin, dibenzocyclooctadiene lignans isolated from *S. chinensis*, induce apoptosis in HL-60 human leukemia cells without altering the intracellular ROS level [11].

In conclusion, the dibenzocyclooctadiene lignans gomisin J and gomisin N, isolated from the fruit of *S. chinensis*, inhibited the Wnt/β-catenin signaling pathway in HCT116 human colon cancer cells. The Wnt/β-catenin inhibitory activities of gomisins J and N were mediated by the disruption of the binding between β-catenin and specific DNA sequences called TCF binding elements. The concomitant decrease in the expression of Cyclin D1, a representative Wnt/β-catenin signaling pathway target gene, was accompanied by a decrease in the expression of Cdk2, Cdk4, and resulted in cell cycle arrest at the G0/G1 phase. Ultimately, gomisins J and N potently inhibited the proliferation of HCT116 cells. Therefore, gomisins J and N, novel inhibitors of the Wnt/β-catenin signaling pathway, may serve as potential candidates of nutraceuticals to be used in the treatment or prevention of human colon cancers.

Acknowledgments

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